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Review

Overall impact of the regulatory requirements for genotoxic impurities on the drug development process

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ABSTRACT

In the last decade a considerable effort has been made both by the regulators and the pharmaceutical industry to assess genotoxic impurities (GTI) in pharmaceutical products. Though the control of impurities in drug substances and products is a well established and consolidated procedure, its extension to GTI has given rise to a number of problems, both in terms of setting the limits and detecting these impurities in pharmaceutical products. Several papers have dealt with this issue, discussing available regulations, providing strategies to evaluate the genotoxic potential of chemical substances, and trying to address the analytical challenge of detecting GTI at trace levels. In this review we would like to discuss the available regulations, the toxicological background for establishing limits, as well as the analytical approaches used for GTI assessment. The final aim is that of providing a complete overview of the topic with updated available information, to address the overall GTI issue during the development of new drug substances.

Contents

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1. Background

The decision to advance new chemical entities (NCE) in clinical trials and eventually to approve new medicines involves a risk/benefit assessment (Lettani and DiFeo, 2005; Gad, 2002). Due to the intrinsic toxicity of drugs its administration to a patient gives rise to a therapeutic effect and at the same time to side effects (Bennett et al., 2005; Rietjens and Alink, 2006). Acceptability of the risk connected with this toxicity depends on the type of disease to be treated, for example whether it is a life threatening condition or not. The duration of the treatment (acute or chronic disease), which corresponds to the duration of exposure to the risk, is the other key parameter to be taken into account in the overall risk evaluation of the therapy. The impurities present in a pharmaceutical product increase the overall risk of the therapy without adding any benefit, the risk being dependent on the number, type and concentration of these impurities (Bouder, 2008; Skett et al., 2007). In general, impurities can be formed: (a) by the degradation of the drug substance (drug related impurities); (b) during the drug substance manufacturing process (process related impurities); (c) during the drug product manufacturing and storage (Ahuja and Alsante, 2003). Among these impurities compounds which are able to induce genetic mutations and chromosomal damage, genotoxic impurities (GTI) may be present. The fact that GTI could cause cancer or genetic anomalies together with the evidence that their effect can be triggered even at very low levels of exposure has given rise to an increasing concern within the pharmaceutical industry and regulatory agencies. Though impurities in drugs cannot be entirely eliminated, substantial efforts must be made to control them at safe concentrations (Ahuja, 2007). The assessment of a safe level of exposure to GTI as well as the approaches used for the identification of these impurities in pharmaceutical products has been the main challenges. The ICH Q3 guidelines summarize the requirements for identification, qualification and control of impurities in drug substances and corresponding formulated products (ICH Q3 guidelines are listed in the reference section along with the other guidelines mentioned in this review). The acceptance criteria for organic impurities in the drug substance are addressed in ICH Q3A (Branch, 2005). In this guideline threshold levels for impurities are listed for reporting, identification and qualification, depending on the daily dose of the drug. Impurities exceeding qualification threshold but present in safety and clinical batches are considered qualified up to the level present in these batches. Also impurities which are significant metabolites are considered qualified. All the other impurities exceeding the qualification threshold must be qualified as summarized in Attachment 3 of Q3A/B. This toxicological investigation includes a genotoxicity study consisting of a bacterial reverse mutation assay (Ames Test) and a chromosomal aberration test. Thus, at least in principle, impurities which could be genotoxic substances are taken into account by the Q3 guidelines. However, since GTI could be carcinogenic to humans even at trace levels, the need to assess them in a pharmaceutical product and, if found, to limit them appropriately in order to guarantee the risk/benefit balance of the therapy, has become a crucial matter.

2. The genotoxic impurity issue

In order to understand whether the genotoxic impurities (GTI) are adequately addressed in the ICH Q3A guideline, it is necessary to consider the identification and qualification thresholds in more detail. The qualification threshold is 0.15% or 0.05%, respectively, if the maximum daily dose of the drug is lower or higher than 2 grams. The Q3A guideline also introduces the concept of TDI (Total Daily Intake) of the impurity, which is expressed in mg/person/day, rather than in concentration. The structure of impurities exceeding the identification threshold (0.10% or 0.05% depending on the daily dose of the drug) must be elucidated. With the identification of the structure it is possible to assess the analytical response factor of the impurity. The response factor is in turn used to calculate the TDI of the impurity. According to Q3A the qualification threshold in terms of TDI corresponds to 1 mg/person/day of the impurity. The concept of PDE (Permitted Daily Exposure), introduced in ICH Q3C to define a pharmaceutically acceptable intake of residual solvents, can be extended to any impurity for setting exposure limits and is calculated as outlined in the guideline. PDE is derived from NOEL (No-Observed-Effect-Level) or LOEL (Lowest-Observed-Effect-Level) obtained in relevant regulatory toxicological animal studies, taking into account corrective factors for extrapolating the dose from animals to humans and the individual variability. While this approach can guarantee the safety of pharmaceutical products containing non-genotoxic impurities it could be not appropriate when dealing with GTI. Indeed for some carcinogenic substances, in particular those which can react with the DNA, it may not be possible to identify a safe level of exposure, since in principle they could cause damage at any concentration. Consequently, at least for some GTI, the PDE as discussed above, cannot be calculated and another different approach to guarantee the pharmaceutical product safety becomes necessary. In addition, the genotoxic effect of several GTI can be elicited at doses well below the limit required by ICH Q3A for identification and qualification of the impurities (1 mg/person/day). Moreover, though for non-genotoxic impurities the testing of the drug substance containing its impurities (at the specification level) is considered an acceptable alternative to the testing of the isolated impurity, the same qualification approach cannot be used in the case of GTI, since in these conditions the test is not sensitive enough even for highly genotoxic chemicals (Cyr et al., 2005). This highlights why a different approach is necessary to identify a GTI and to establish corresponding limits. The issues discussed above triggered a large debate in the pharmaceutical world. A draft position paper on the limits for genotoxic impurities was published by the European Committee for Proprietary Medicinal Products in 2002 (CPMP, po-

sition paper on the limits of genotoxic impurities, London, 2002; finalized as the Guideline on the Limits of Genotoxic Impurities and effective from January 2007). In 2003 an EMA workshop was held in London on this topic (Kasper et al., 2004). Several papers were also published focusing on this issue (Bercu et al., 2009; Humfrey, 2007; McGovern and Jacobson-Kram, 2006; Kirkland and Snodin, 2004; Leblanc et al., 2004). In 2005 PhRMA (Pharmaceutical Research & Manufacturing Association) published a contribution (Muller et al., 2006) focusing the problem of controlling genotoxic impurities during clinical development, since in the EMA position paper this topic was not discussed. Later on, in 2008, EMA issued a Question & Answer document (updated in December 2009) to provide clarification and harmonization of the guideline. In March 2008 the European Directorate for the Quality of Medicines & HealthCare (EDQM) issued a document on potential genotoxic impurities (Monograph No. 2034) to point out how to cope with these impurities during elaboration and revision of monographs. In December 2008 CDER issued a draft guidance for industry: "Genotoxic and Carcinogenic Impurities in Drug Substances and Products: Recommended Approaches" with the aim of informing pharmaceutical manufacturers about the FDA opinion on the subject and to integrate the ICH Q3 guidelines with a part addressing the genotoxic and carcinogenic impurities.

3. The EMA and FDA guidelines on genotoxic and carcinogenic impurities

3.1. The EMA guideline on the limits of genotoxic impurities

The EMA guideline on the limits of genotoxic impurities defines the framework on how to deal with GTI in new drug substances. It only applies to new applications for already commercialized products and for manufacturing process changes, where there is no reasonable assurance that no new or higher levels of GTI are introduced. Thus, the guideline does not need to be applied retrospectively to authorized products unless there is a specific cause for concern. The problem of addressing the GTI issue for already registered products is discussed more in depth in the EDQM document. The toxicological assessment of GTI and how to establish corresponding limits for new drug substances are the critical topics discussed in the EMA guideline.

3.1.1. Classification of genotoxic impurities

The guideline suggests distinguishing the genotoxic compounds between those directly interacting with DNA (alkylating and intercalating agents) and those acting through other mechanisms. Compounds in the first group are the source of main concern. For these substances a threshold-related mechanism cannot be recognized since they could cause damage at any concentration. Conversely, for compounds in the second group a threshold-related mechanism can usually be demonstrated. For compounds with a demonstrated threshold-related mechanism, acceptable exposure levels can be established calculating the PDE from NOEL or LOEL, as discussed for non-genotoxic impurities. Thus, for the determination of acceptable levels of exposure to a GTI, it is important to know whether there is or not sufficient experimental evidence for a threshold-related mechanism. However, this scientific approach of correlating the risk of exposure to a GTI with its mode of action implies appropriate compound specific toxicological information. This information is often lacking for the majority of the substances used as reagents or intermediates in the synthetic processes (SCHER, 2009, Risk assessment methodologies and approaches for genotoxic and carcinogenic substances). Thus, while for some well known GTI it is possible to recognize the mode of action, it can require a considerable effort to provide the sufficient experimental evidence to establish whether an unstudied compound acts through a threshold-related mechanism or not.

3.1.2. The ALARP principle

In the case of unavoidable GTI with a non-demonstrated threshold-related mechanism, the guideline suggests to control their level according to the ALARP (as low as reasonably practicable) principle. The ALARP principle is widely used in industry when safety-critical systems are involved and when a balance between risk and benefit is necessary. In this case the ALARP principle is based on setting a balance between the need to reduce the impurity concentration at the lowest possible level and the practical feasibility of this reduction. The assessment of what is practically feasible should involve the evaluation of process capability to remove the impurity and the development of suitable analytical methods for its quantification. The guideline specifies that the methods for GTI detection need to conform to the state of the art techniques. On the other hand, it is not straightforward to estimate consistently which level of an impurity corresponds to the practicable process capability for its removal. In addition, even if the ALARP principle clearly expresses the need to focus all efforts on reducing the concentration of the GTI at a level "as low as reasonably practicable", this does not guarantee in itself that the achieved low concentration of the impurity is safe. Thus, according to the guideline, the evaluation of acceptability for the determination of acceptable levels of exposure to GTI with a non-demonstrated threshold-related mechanism includes a pharmaceutical and toxicological assessment.

3.1.3. Pharmaceutical assessment

The genotoxic potential of the impurities is evaluated in the pharmaceutical assessment with the hazard identification. At this stage of the risk assessment, potential genotoxic impurities (PGI) and GTI are identified by a careful analysis of both the degradation products and the manufacturing process, taking into account reagents, intermediates and by-products. The identification of GTI is mainly based on available toxicological data, while PGIs can be identified using structural alerts (structural similarity with known genotoxic substances). The *in vitro* assay (Ames test) for genotoxicity can then confirm if a PGI is actually a GTI. When the presence of a GTI is established, an explanation why no alternative to the use/formation of this impurity is possible (including alternate synthetic routes) should be provided as part of the pharmaceutical assessment. The technical effort aimed at reducing the GTI level according to ALARP should be documented. This latter part of the pharmaceutical assessment could give rise to some practical applicability issues. Due to their intrinsic high reactivity, reactants and intermediates used in manufacturing processes are often genotoxic substances and it could seem impossible to avoid their use. However, during process development it is possible to evaluate whether the same reaction can be appropriately obtained using less toxic reagents. In addition, an in-depth knowledge on the fate of the GTI during the process can help to increase the process purging capability, thus reducing the level of these impurities in the final product. Changing or modifying the synthetic route can be more or less feasible depending on the development status of the drug. During development it is common practice to evaluate several possible different routes for the synthesis of the drug substance. Often the route used by medicinal chemists in drug discovery is aimed at being the fastest, in order to provide the highest number of compounds in the shortest time, without taking into account yields and purification issues. At the stage of candidate drug selection, different synthetic pathways are explored in order to identify a scalable route taking into account both yields and process operability issues. At this point the evaluation of the possible routes also in terms of GTI presence is feasible. Examples

of this strategy aimed at avoiding or reducing GTI presence, based on the use of a different synthetic pathway or alternative reagents were reported (Robinson, 2010).

3.1.4. Toxicological assessment

A complete risk assessment consists of hazard identification, hazard characterization and exposure assessment. In hazard identification chemicals are evaluated on the basis of available chemical-specific knowledge leading to a qualitative assessment about possible impacts on safety. Then hazard characterization (dose–response) and a quantitative estimate of the intake that would not give rise to a significant risk of adverse effects would complete the safety assessment. Although for impurities with a threshold-related mechanism and data from long-term carcinogenicity studies, a quantitative estimate of a safe intake (PDE) is possible, for GTI with limited or no data at all, this approach cannot be applied. Despite Ames and *in vitro* chromosomal aberration tests are used to confirm whether a PGI is actually a GTI, it is not considered appropriate to extrapolate limits of exposure to a GTI using the data obtained from these tests. Since these assays are established as qualitative tests (yes/no) and they are not suited for dose extrapolation. Thus, when a compound is positive in these tests an approach for establishing the exposure level related to a negligible risk becomes necessary.

3.1.5. The threshold of toxicological concern

The concept of threshold of toxicological concern (TTC) is discussed in the guideline. In this context the TTC represents an estimate of a daily exposure level of most carcinogens which would cause less than one in a 100.000 lifetime risk of cancer: this exposure level corresponds to 1.5 μg/person/day. According to the guideline, limiting the GTI level at, or below the TTC obviates the need for further qualification to support marketing authorization. As discussed in the Q&A document, when the concentration of the impurity does not exceed the TTC limit it is not necessary to apply the ALARP principle, unless the impurity belongs to the class of highly genotoxic compounds discussed below. More in general, the TTC is a risk assessment tool, based on the principle of establishing a human exposure threshold value for chemicals, below which there is a very low probability of adverse effects. In the absence of substance-specific toxicological data, an extensive and time-consuming toxicology assessment would be necessary in order to complete the risk characterization. Thus, the identification of a general exposure limit for safety, to be used as a substitute for substance-specific information was the task of several works. The TTC concept appeared first as ToR (Threshold of Regulation) and was applied by FDA to manage low doses of exposure in the control of potential carcinogenic substances in food packing materials (Munro, 1990). The analysis of the chronic dosage rate for 343 carcinogens (Gold et al., 1991) made it possible to estimate, by extrapolation to a distribution of 10^{-6} risk of developing cancer, the value of 1.5 μg/person/day. Cheeseman (Cheeseman et al., 1999) extended the ToR concept to the incorporation of acute and short-term toxicity data and to the results of genotoxicity testing and structural alerts, to identify potent and non-potent carcinogens in an evaluation which involved 709 rodent carcinogens from the RTECS database (Registry of Toxic Effects of Chemical Substances). The concept of TTC was further elaborated in the following years (Munro et al., 1996; Kroes et al., 2000; Kroes, 2005). It was adopted by WHO for the control of flavoring substances and cosmetics (Munro et al., 1999; Kroes et al., 2007). The use of a more conservative TTC value of 0.15 μg/person/day in a further evaluation carried out by Kroes et al. (2004) was proposed, when dealing with very potent carcinogenic compounds. Thus, the TTC concept has evolved as a pragmatic risk assessment tool, based on the principle of establishing a human exposure threshold value for all chemicals. This limit should guarantee a very low probability of an appreciable risk to human health, when the intake is below such a threshold, avoiding extensive safety evaluations. The use of the TTC concept for the assessment of acceptable limits for GTI in pharmaceuticals is the consequence of what is discussed above.

3.1.6. TTC applicability

According to the EMA guideline, the application of 1.5 instead of 0.15 μg/person/day as the TTC limit for GTI in drug substances is justified, since for pharmaceuticals a therapeutic benefit exists. The limit of the impurity can then be calculated from the TTC and the daily dose of the drug. The TTC value is not applicable when the GTI belongs to the class of aflatoxin-like compounds or N-nitroso or azoxy compounds. For these highly genotoxic compounds the lower limit (0.15 μg/person/day) must be applied, alternatively the risk assessment requires compound-specific toxicity data. According to the guideline, limits higher than the TTC can be applied: (a) in the case of short-term exposure; (b) for treatment of life-threatening conditions; (c) when life expectancy is less than 5 years; (d) when the GTI is a known substance and human exposure will be much greater from other sources (i.e. foods); (e) when the genotoxic impurity is also a significant metabolite. For anti-cancer therapeutics intended to treat patients with late-stage or advanced cancer, less stringent criteria can be applied (ICH S9; March 2009). Finally, there may be reasons to deviate from TTC and positive results from *in vitro* studies, when there is lack of *in vivo* relevance of the *in vitro* results that can be based on the weight-of-evidence approach (WOE).

3.1.7. Staged-TTC

The EMA guideline introducing the TTC concept considered the administration of the drug as a lifelong treatment. How to cope with the GTI topic during clinical development, where the duration of the administration of the drug is limited, was not specifically discussed by the guideline. In 2006 PhRMA Genotoxic Impurities Task Force published a paper (Muller et al., 2006), where the concept of using a "staged" TTC approach was proposed, in order to commensurate the effort done to limit impurities with the effective risk at each clinical phase. The staged TTC approach is based on the fact that even maintaining the concept of TTC, different limits can be calculated when a timeframe shorter than a lifelong treatment is considered. Staged TTC limits were calculated using a linear extrapolation from the more conservative TTC value of 0.15 μg/person/day, depending on the duration of the clinical trial. Thus, the TTC daily dose of 1.5 μg/person became, respectively, 10, 20, 40 or 120 μg/person, for decreasing the duration of exposure from 12 months to 1 month. The limit of 0.15 μg/person/day was used taking into account that healthy volunteers could be exposed to risks without any therapeutic benefit during the trial. This proposal was accepted by EMA (Q&A document), provided that a dose rate correction factor of 2 was used to take into account deviations from linearity. Thus, 120 μg/person/day became acceptable as a single dose administration and 60, 20 and 10 μg/person/day were the accepted exposures, respectively, for 1, 3 and 6 months. The dose of 5 μg/person was the acceptable daily intake for clinical trials up to 1 year, while for longer exposure times the accepted limit was 1.5 μg/person/day.

3.1.8. Limits for multiple genotoxic impurities

The EMA Q&A document sets the criteria to establish the limits when more than one GTI is present in the same drug substance. The TTC limit is applied to each individual impurity when the impurities are structurally unrelated. When GTI are structurally related, the limitation of the sum of the exposures at 1.5 μg/person/day is recommended. This more stringent requirement for structurally similar impurities, which should act through the same

mode of action, is supported by the possibility of an increased cancer risk due to the concurrent exposure. However, assessing multiple impurities in the low ppm range can constitute a significant analytical problem. Thus, the possibility of demonstrating that the concentrations of the GTI are sufficiently below the TTC value can be accepted on a case-by-case basis. Key factors to be taken into account are: the daily dose of the drug, the therapeutic indication, the possibility for the process to remove these impurities, and issues related to the analytical control. How to manage additive risks in genotoxicity is still a challenging issue, due to the limited available information about toxicological synergies. At least two points deserve discussion in the above approach: (1) the relationship between structural similarity and the mode of action, (2) how an increased risk due to the multiple GTI effect is significant when the level of each GTI is at the TTC level (Bercu et al., 2008). Aspects dealing with the control of multiple structurally related GTI, including the two issues above were recently discussed (Elder and Harvey, 2010).

3.2. The FDA guidance on genotoxic and carcinogenic impurities

This guidance was intended as a supplement to the ICH Q3 guidelines, in order to provide specific recommendations for appropriate qualification of impurities with known or suspected genotoxic or carcinogenic potential. The guidance applies to clinical development (IND), marketing (NDA), biologics license (BLA) and abbreviated new drug (ANDA) applications. The FDA guidance has to be applied to already approved products only if a specific safety concern exists. The guidance is also applicable to already marketed drugs for supplemental applications which could involve an increased carcinogenic risk, for example, new indications, changes in dose regimen or longer duration of use. Finally, the guidance applies to changes in the manufacturing process or in formulation. The guidance describes the Agency point of view on genotoxic and carcinogenic impurities, recommending the approaches to be used for assessment, prevention and reduction of GTI in drug substances and the corresponding acceptable levels. The EMA guideline is discussed in the background and the policy for setting limits for marketing application is considered acceptable.

3.2.1. Assessment of genotoxic potential

The FDA guidance takes into account that in most cases appropriate data for establishing whether a genotoxic compound acts through a threshold-related mechanism are not available. Impurities exceeding the ICH Q3A qualification threshold are firstly taken into account. If appropriate toxicological data are lacking, screening of the isolated impurity in the standard *in vitro* battery for genotoxicity is recommended. The option to conduct these studies by spiking the drug substance with the impurity is considered acceptable, when it is not feasible to obtain the impurity in the necessary amount. The level of the impurity must not exceed the corresponding level in the batch used for genotoxicity assessment and this level must be consistent with clinical and stability batches. The impurities exceeding the identification threshold should be evaluated for genotoxic potential, using available data or by a computational toxicology assessment. Those with a structural alert can be confirmed to be GTI using the Ames test. If there is any indication of the possible presence of a GTI, all appropriate investigations should be made to address the corresponding safety concern. Thus, the actions required to address the risk assessment are substantially the same in both the EMA and FDA guidelines, though a more pragmatic approach characterizes the FDA guidance.

3.2.2. GTI: prevention of formation and reduction of the level

Once genotoxicity has been confirmed for an impurity, or available information points to a carcinogenic potential, all feasible technical effort should be made to prevent its formation and to limit its presence in the drug substance. If, despite these efforts, appropriate limits are not reached during clinical development, the guidance suggests carrying out a characterization of the genotoxic and carcinogenic properties of the substance, in order to establish a limit compatible with the specific risk. The need to develop appropriate analytical methods for identifying and monitoring GTI at the appropriate threshold levels is also pointed out.

3.2.3. Limits for genotoxic and carcinogenic impurities

For marketing applications the TTC limit (1.5 μg/person/day) is considered acceptable, with the same exception for highly potent carcinogenic compounds as discussed above. The guidance also recommends taking into account the higher cancer susceptibility in pediatrics and a correction factor of 10 is applied in the case of children up to 2 years of age, while a correction factor of 3 is used when the exposure occurs between 2 and 16 years. The guidance considers the case for non-oral route of administration such as dermal or ophthalmic, in these cases the TTC can be applied or the authorities can be contacted to discuss a more specific approach. The issue of multiple impurities is also addressed. It is recognized that limiting each impurity at the TTC level does not guarantee an appropriate combined cancer risk. Particularly, when a synergy is expected, due to a common mode of action, the total exposure should be appropriately evaluated. The staged TTC approach is considered suitable for controlling GTI during clinical development and the same limits above discussed for the EMA guideline are recommended.

4. Focus on the Ames test and the toxicological assessment

4.1. Ames test

The Ames test is used as the key assay for confirming the genotoxic potential of an impurity highlighted as PGI by a structural alert, since many chemicals that are mutagenic in the Ames test are carcinogens in rodents. According to the EMA Q&A document, a positive Ames test confirms that the TTC limit must be applied, while a negative Ames test, conducted according to regulatory standards, overrules any structural alert, and the limit for the impurity can be established according to ICH Q3A criteria. Thus, genotoxic carcinogens can be identified by combining the use of structural alerts with the Ames test (Ashby and Tennant, 1988; Dobo et al., 2006). The Ames test is considered to be predictive for genotoxicity since most carcinogens are positive in this test, with a very low incidence of false negatives when the appropriate concentration of the tested substance is used (Ames et al., 1973a,b). The most common exception are carbamates: genotoxicity of these compounds is not detected by the Ames test and, therefore, a mutagenicity test in mammalian cells is necessary. The detection limit for most important mutagens in the Ames test is 250 μg/plate (Kenyon et al., 2007). If an impurity is present at 0.15% and the drug substance is tested up to 5 mg/plate, even powerful mutagens such as 9-aminoacridine and methyl methanesulfonate routinely used as positive controls in the assay would pass undetected. Hence the need to test the isolated impurity. Due to this sensitivity issue, when possible, the Ames test is conducted with a dose of up to 5000 μg/plate. It should be taken into account that the upper test concentration is limited both by the solubility and cytotoxicity of the tested compound. When no cytotoxicity is observed, the upper limit of the experiment is the lowest concentration which gives rise to precipitation. Recommendations

for *in vitro* genotoxicity test execution, including the Ames test, can be found in the ICH S2(R1) guideline. According to the regulatory protocol (OECD Guidelines for Genetic Toxicology) the Ames test is carried out within the concentration range discussed above (250–5000 μg/plate), using at least 5 different strains of *Salmonella typhimurium*, optionally one of them can be substituted with one strain of *Escherichia coli*. These tests have to be conducted with and without metabolic activation. Equivocal or weak positive results require the test to be repeated. Consequently, for an Ames test done according to the regulatory requirements, including the work for the experimental set-up, the execution of the protocol, and for confirmatory tests, a significant overall amount (up to 1 g) of the impurity is necessary (about 3 g for the whole regulatory standard battery). The procedure for the Ames test was recently revised in order to reduce the amount of the test compound (Kamber et al., 2009). A miniaturized version of the Ames test which requires no more than 300 mg (Flamad et al., 2001) and several mini-Ames assays which use about 10 mg of the substance, are available nowadays for preliminary genotoxicity screening. Sometimes the Ames test gives positive results which do not correspond to genotoxicity and carcinogenicity in mammals. This is due to protective or elimination mechanisms and/or to different cell control mechanisms effective in mammals but not in bacteria (Kirkland et al., 2007; Thybaud, 2007).

4.2. More in-depth studies

A positive Ames result does not necessarily mean that the substance is a mutagen in humans. In the case of uncertainty, the standard battery for mutagenicity can be applied (Kirkland et al., 2006). According to the ICH S2 (R1) guideline (currently at step 3) the standard battery consists of:

- Assessment of mutagenicity in a bacterial reverse mutation assay.
- A cytogenic test for chromosomal damage (the *in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or an *in vitro* mouse lymphoma tk gene mutation assay.
- An *in vivo* test for genotoxicity, generally a test for chromosomal damage using rodents' hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.

A second option is considered equally suitable:

- Assessment of mutagenicity in a bacterial reverse mutation assay.
- An *in vivo* assessment of genotoxicity with two tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second *in vivo* assay.

The *in vivo* tests described as second option could be used as follow up tests for the weight-of-evidence (WOE) in assessing *in vitro* results. Though induced chromosomal and gene mutations play a role in carcinogenesis, it is widely accepted that *in vivo* mutation assays are more relevant to the human risk than are *in vitro* assays (Johannsen, 1990). The need of an appropriate interpretation of the *in vitro* data is also discussed in the FDA guidance "Recommended approaches to integration of genetic toxicology study results". In this document the Agency suggests three options for further studies in the case of an *in vitro* positive response. The first one is the WOE approach, which consists of a critical review of the available data. The results could suggest the lack of a real genotoxic hazard even in the presence of one or more *in vitro* positive tests. Attention during this review should be paid to the relevance of the dose which elicited the effect, to the biological significance of the effect seen and to the consistency of what was found in one test compared with the other. Second option is to study the mode of action of the GTI. Knowledge of the mechanism could demonstrate that a certain positive response, obtained under non-physiological exposure conditions, is not relevant to human risk. The existence of a threshold or the presence of a mechanism not expected to be effective *in vivo* can also be demonstrated by these studies. The last recommended option is to perform additional supportive studies *in vivo*, such as bone marrow cytogenetic studies, micronucleus induction and/or chromosome damage assessment. Finally, the evaluation of mutagenicity in potential target tissues using transgenic rats or mice is suggested. The strategies accepted by the regulatory agencies to evaluate the carcinogenic potential were comparatively reviewed (Cimino, 2006). The work done for assessing acceptable limits for ethyl methanesulfonate in Viracept was recently reviewed (Walker et al., 2009) and represents a good example of the effort necessary to establish the carcinogenic potential by additional studies. In the ICH S2(R2) guideline tests to confirm *in vitro* results are reported, including: the DNA strand break assays such as the "Comet" assay, the mouse mutation assay, and the DNA covalent binding assay. The "Comet" assay, or Single-Cell Gel Electrophoresis (SCGE) technique is a versatile and sensitive method to measure DNA damage (single and double-strand breaks) in eukaryotic cells (Collins et al., 2008). This test can be used as a supplementary assay for substances with positive results from *in vitro* tests. It is useful for the understanding of the mechanism and it has a number of advantages over the other tests, such as the unscheduled DNA synthesis (UDS) method (Williams, 1977). These advantages are: (a) the fact that the Comet test can be applied to a greater number of tissues; (b) the broader spectrum of primary DNA lesions which can be detected with this test; (c) the limited number of cell samples required. The biological relevance of the results highlighted by the Comet assay and the application of this test in genotoxicity assessment were recently reviewed (Brendler-Schwaab et al., 2005). The development of transgenic rodent (TGR) mutation models has provided a useful tool to detect, quantify and sequence mutations in a range of somatic and germ cells. A comprehensive review which discusses the potential use of TGR mutation models in a regulatory context and compares their performance in genetic mutation assessment with available non-transgenic assays was published (Lambert et al., 2005). However, the current standard approach for assessing carcinogenic potential and to estimate the potential human cancer risk still involves carrying out a carcinogenicity study in rodents for all their life (2 years) and with the high dose set to the maximum tolerated dose (MTD) of the tested chemical. A strategy for classifying carcinogens that are acting through genotoxic, cytotoxic, or mitogenic pathways can be applied during this study. Thus the study can highlight whether the chemical (or its metabolite/s) has direct genotoxic potential resulting from DNA reactivity or clastogenicity, allowing for the identification of the mode of action of the substance (Butterworth et al., 1995). In conclusion, although the TTC approach represents the more straightforward and less resource-consuming approach for establishing limits for GTI, several methodologies are available to confirm and quantify the genotoxic potential, as well as to identify the mechanism and to establish substance-specific limits, when this is possible.

5. Identification, classification and limits for genotoxic impurities

Degradation products and process-related substances are reviewed during the pharmaceutical assessment in order to evaluate

their genotoxic potential. Starting materials, reagents and intermediates may contain functional groups that could react with the DNA. The identification of PGI or known GTI can been achieved using publicly accessible data or specialized software.

5.1. Available databases

Genotoxic and/or carcinogenic properties for a number of substances are reported by Potency Database, CPDB (http://potency.berkeley.edu). This database reports results from technical reports issued by the National Cancer Institute for several chemicals. Information about functional groups and compounds that can react with DNA (Benigni, 2004) can be found in databases such as TOXNET (http://toxnet.nlm.nih.gov), NIOSH (National Institute for Occupational Safety & Health; http://www.cdc.gov.niosh), GESTIS (http://www.dguv.de/bgia/en/gestis), Discovery Gate (Symx) and PharmaPendium (Elsevier).

5.2. Structural alerts

When substance specific data on genotoxicity/carcinogenicity are not available, the impurities can be screened for the presence of structural alerts. The identification of structural alerts is important both for assessing substance related risks and for understanding the mode of action (Snodin, 2010). The structural alerts are molecular substructures or functional groups related to the potential mutagenic/carcinogenic properties of the substance. They can be identified by a structure–activity relationship (SAR) analysis between the chemical structure/physicochemical properties of the compound and the biological effects. Ashby and Tennant analyzed the relationship between the structure and the mutagenic/carcinogenic properties for several chemicals and identified structural alerts which have been used to predict the genotoxic potential of a compound (Ashby and Tennant, 1991). The advances in computational potential, the widespread use of computers for SAR analysis in medicinal chemistry, as well as the need for a fast first pass screening to assess toxicity, prompted the development of computational methods for toxicological assessment. These methods are based on structure similarity and quantitative structure–activity relationship (QSAR) models (Green, 2002; Dearden, 2004; Yang et al., 2006).

5.3. Expert systems

Computer modelling programs such as DEREK (Deductive Estimation of Risk from Existing Knowledge; http://www.chem.leeds.ac.uk/luk/derek; Ridings et al., 1996), MCASE (Multi-Computer-Automated-Structure-Evaluation; http://www.multicase.com) and TOPKAT (http://accelerys.com/products/topkat) are widely used for a structure-based search to predict mutagenic or carcinogenic potential for compounds without experimental data (Mayer et al., 2008; Kruhlak et al., 2007). *In silico* predictive models for genotoxicity can be divided into two general categories: (a) programs designed to capture and automate rules and decision trees based on human expertise (rule-based expert system); (b) programs combining human expert decisions with statistical correlative approaches (QSAR models). DEREK is an example of the first category while MCASE (MC4PC) and TOPKAT are examples of the second one. The DEREK system makes its predictions based on a series of rules contained in its knowledge base. Each rule describes the relationship between a structural feature (toxicophore) and its associated toxicity. When a structure is processed the program compares structural features in the target compound with the toxicophores described in its knowledge base. A rule is activated when the toxicophore is recognized in the target structure and the toxic effects expected for the compound are predicted. Mutagenicity, carcinogenicity and teratogenicity can be estimated by DEREK, along with other toxic effects (Green et al., 1999; Marchant et al., 1996). Another rule-based system able to predict carcinogenicity is OncoLogic (EPA OPPT, LogiChem, Inc.). Conversely, a QSAR model designed to estimate genotoxicity correlates the chemical structure, given in terms of molecular descriptors, to the compound biological properties. Used descriptors are: chemical substructures, topological parameters, LogP, electronic parameters, as well as combinations of different classes of descriptors (geometric, electronic, polar surface area, topological). Algorithms utilized in these predictive models are: machine-learning ranking (MLR), partial last squares (PLS), hierarchical QSAR and inductive logistic programming (ILP). MCASE (MC4PC) is an example of statistical correlative program that contains several predefined modules for mutagenic, carcinogenic and teratogenic properties. The modules are designed to capture SAR information from several chemicals, pharmaceuticals and natural compounds. The program uses an algorithm that reduces each compound into all possible 2–10 atom fragments, creating a training set which relates these fragments to their biological action. The compound of unknown toxicity is then fragmented into all possible 2–10 atom subunits, which are compared with the training set and its associated biological activity to provide an estimation of toxicity (Saiakhov and Klopman, 2008). TOPKAT and MDL-QSAR (Contrera et al., 2008) are further examples of QSAR based expert systems. The performance of these rule-based and QSAR systems to assess genotoxic and carcinogenic properties was comparatively evaluated using marketed drugs as test compounds (Snyder et al., 2004; Snyder, 2009). The coordinated use of at least two expert systems is recommended by ICSAS (Informatics and Computational Safety Analysis Staff, FDA) for the screening and reconfirmation of the results obtained in the prediction of carcinogenicity. A study which describes the multiple use of four QSAR programs and one expert knowledge-based system to predict carcinogenicity in rodents was published (Matthews et al., 2008). Results demonstrated that the four QSAR programs were complementary, each detecting different profiles of carcinogens. Accepting any positive prediction from two of these programs gave a better overall performance than either of the single programs alone. A computational method to predict structural alerts arising from drug substance degradation was recently reported (Raillard et al., 2010). The CambridgeSoft Pharmaceutical Drug Degradation Database was used to analyze the frequency and the kind of structural alerts present in degradation products. Taking into account the degradation pathways leading to alerting structures, it was possible to identify a method for the prediction of features in the drug substance able to generate degradation products with structural alerts. When the assessment is done with a software considered suitable by the Regulatory Authorities, the absence of a structural alert in an impurity is sufficient to conclude there is no genotoxic potential for that impurity and further actions are not necessary. Results obtained by MCASE and DEREK are generally accepted by the Authorities (Q&A document).

5.4. Setting limits for GTI

Based on the discussion in the previous sections, the limits for GTI can be established according to the TTC or the PDE, on a case-by-case approach (Kirkland and Snodin, 2004; Leblanc et al., 2004). In both the EMA and FDA guidelines a decision tree for assessment of GTI acceptability is featured. The EMA guideline considers firstly if the GTI acts through a threshold-related mechanism or not. On the other hand, the FDA guidance is based upon the impurity identification and its presence at a level higher than the qualification threshold, or the existence of a structural alert. When the presence of a GTI has been confirmed both the

guidelines consider the possibility of preventing its formation/presence. If prevention is not feasible, the EMA guideline applies the ALARP principle, and then evaluates if the obtained level of the impurity is higher or lower than the TTC. If it is higher, an evaluation of the acceptability of this level must be carried out. The FDA guidance considers the option to apply the TTC or to conduct further genotoxicity studies when it is not possible to avoid the GTI presence. Even though at different steps of the decision tree, both the guidelines consider the need to assess the mode of action of the GTI. When an experimental threshold-related mechanism cannot be proved the TTC limit must be applied.

5.5. GTI categories and limits

On the basis of the available data, impurities identified during the pharmaceutical assessment can be classified in terms of the related risk level, according to five categories (Dobo et al., 2006; Muller et al., 2006):

- Category 1: compounds with literature data (at least in animal models) which provide evidence for carcinogenicity.
- Category 2: compounds which are known mutagens with unknown carcinogenic potential (positive in the Ames test but without evidence for *in vivo* carcinogenicity).
- Category 3: compounds with a structural alert (not shared with the drug substance) not confirmed by the Ames test.
- Category 4: compounds with a structural alert shared with the drug substance. The impurity is considered qualified in this case since genotoxicity of the drug substance is characterized.
- Category 5: compounds without structural alert.

Limits for the identified and classified GTI can then be established as outlined below.

- For compounds in Category 1: the first action is to eliminate them from the process. If this is not possible the TTC limit must be applied.
- For compounds in Category 2: it can be decided to use the TTC limit or to do a specific toxicological risk assessment; if a threshold-related mechanism can be demonstrated the PDE limit can be calculated.
- For compounds in Category 3: the TTC limit can be applied or the actual genotoxic potential can be assessed by the Ames test. If the test is positive the TTC limit can be used or a more specific toxicological assessment can be carried out. As discussed for compounds in Category 2, if a threshold-related mechanism can be demonstrated the PDE limit can be calculated. If the Ames test is negative the Q3A criteria for non-genotoxic impurities are applied. Q3A criteria also apply for compounds in Categories 4 or 5. When the impurity is in Category 2 or 3, the option to apply the TTC or to study the toxicology of the compound more in-depth can be considered. The staged-TTC limit can be applied during clinical development, in the meanwhile the increased knowledge about the process can confirm whether to control the impurity at the TTC level is an achievable task or not. If the control of the impurity at the TTC level turns out to be not practicable, it could be worthwhile doing a further toxicological characterization. This characterization can be done by a step-by-step approach. The confirmation of the Ames result and corresponding biological relevance are addressed firstly. Experiments aimed at assessing the GTI mode of action can be carried out after the confirmation of genotoxicity. The limit for the impurity can then be assessed by appropriate *in vivo* studies, when a threshold-related mechanism is proved.

6. Genotoxic impurities: analytical control

Taking into account the TTC limit (1.5 μg/person/day) and the corresponding levels of the GTI in the drug substance, it can be calculated that for a daily dose of 1 g/person the limit for the GTI is 1.5 ppm and for a daily dose of 100 mg/person the corresponding limit is 15 ppm. This translates into target limits for GTI detection and quantification at levels of about 1 ppm, that is almost 500 times lower than those for classical impurity analysis (1 ppm vs. 0.05%). These limits represent a challenge for analytical method development, since the implementation of sensitive and selective methods for various GTI at trace level is all but straightforward (Skett et al., 2007). Since GTI can be degradation products of the drug substance, intermediates, reagents or by-products of the manufacturing process, they comprise a wide group of different substances, some of them highly reactive. Taken together the heterogeneous nature, the target level at which GTI need to be assessed and the problems related to the matrix make the whole issue of analytical control a complex problem. A further complication stems from the fact that quality control units of manufacturing sites may not be equipped with mass spectrometers and other advanced analytical instruments that are necessary for the task. In addition, the analysts may not be trained in operating such instruments and not skilled to deal with complex separation procedures. As an overall strategy it is appropriate to split the problem into two parts:

(a) During pre-clinical or early-clinical stages, flexible and sensitive methods able to assess GTI at the TTC limit will be developed in R&D labs. During development these methods should be used not only to assess GTI in the drug substance, but also to monitor the concentration of these impurities at each manufacturing step.
(b) When the process knowledge is mature enough for a technology transfer to production facilities, both the increased knowledge of the GTI analytical characteristics and of the target limits (in the drug substance and intermediates) will allow for development of more appropriate and robust analytical methods (Pierson et al., 2009).

6.1. Strategies for analytical method development

Several strategies have been devised to systematically address the problem of GTI analytical method development. These strategies may depend on the structure of the analytical unit and they can vary from company to company. For example, in big companies there is a need for analyzing several GTI in a wide range of drug substances and, therefore, necessary standard and general methods have to be used, since in these companies the GTI issue has to be addressed simultaneously for a number of development projects. According to a recent paper (David et al., 2009) the analytical development activity should begin with considerations about the volatility/stability of the GTI and GC is the first option to be taken into account. As a second option, LC can be used when the GTI is thermally unstable or not volatile (a decision tree for the implementation of this approach is reported, David et al., 2009). Then, the impurities are assessed by means of general analytical methods, tailored for different classes of GTI, such as: organohalides, sulfonates, halo-alcohols, epoxides, aziridines, arylamines, aminopyridines, aldehydes, ketones, hydrazines, etc. Conversely, a strategy based more on development of fit-for-purpose methods is driven by the need to define the purpose of the analytical method (QC release or research) (Sun et al., 2010a). The whole analytical procedure can be disassembled into three parts or operations: sample preparation, separation and detection. According to this ap-

proach, in addition to the GTI properties also the sample matrix is essential to the selection of the techniques to be used for each part. Each operation is potentially interconnected with the others, since the choice of the method for sample preparation can dictate the corresponding separation and detection techniques. For this reason the three parts should be evaluated holistically. Several methods can then be identified depending on the type and stability of the analyte and categorized with respect to their sensitivity and complexity in order to select the appropriate method for testing. A review of procedures for sample preparation and GTI analysis on the basis of the functional groups which can be present was recently published (Liu et al., 2010). In small companies both the number of compounds under development and the analytical resources are limited in comparison to big companies. In this case an analytical development strategy more focused on the fit-for-purpose approach could be more appropriate. Though the known methods used for GTI analysis should always be taken into account, changes in the matrix make it sometimes difficult to properly utilize an already known method for a certain GTI for a different drug. Over-elaborated procedures for sample preparation are often necessary on changing the matrix and this could highly affect the method performance. Furthermore, a dramatic reduction in sensitivity and/or accuracy can often occur when a reported method for a GTI is directly applied on a different matrix. The available literature on GTI and trace analysis are continuously increasing. In most cases, capitalizing on this information makes it possible to identify appropriate analytical approaches to adequately address many problems related with GTI analysis.

6.2. Detection and sensitivity

The specification limit for an impurity is the key factor in determining the sensitivity requirements for the analytical method and can often dictate the appropriate technique for detection (Olsen and Baertschi, 2004). At the level of 100 ppm, LC with UV detection and GC with flame ionization detection are often adequate. In the range of 10–1 ppm or lower, even though in some cases UV detection can still be applied with success (Soman et al., 2009), hyphenated MS techniques such as LC–MS and GC–MS are by far the most appropriate techniques. These techniques, due to sensitivity and selectivity, have been widely used in GTI analysis (An et al., 2008; Li and Sluggett, 2005; Pereira et al., 2008; Lee et al., 2010). Electrospray ionization (ESI) is an ionization method widely used in LC–MS. Alternatively, atmospheric pressure chemical ionization (APCI) can be used, both in the positive or negative ion detection mode. ESI or APCI is widely utilized also with GC–MS. Currently the most popular mass separation devices are quadrupoles (either single or triple quadrupole) and ion traps. Quadrupole mass analyzers are standard for quantitation and selected ion monitoring (SIM) and multiple reaction monitoring (MRM) are the most commonly used detection modes. SIM is the typically used mode with a single quadrupole, while MRM requires the triple quadrupole. Inductively Coupled Plasma Mass Spectrometry (ICP-MS), in addition to organometallic compound analysis, can also be used for substances containing nitrogen, sulfur, phosphorus and halogens. The sensitivity is quite high, since ppb level detection can easily be reached with this detector (Carr et al., 2008; Huang et al., 2006). Even though MS-detection is considered a "general" method in GTI analysis, it may depend on the ionization properties of the analyte and often trace level analysis of neutral GTI is hampered by poor ionization efficiency. Two approaches can be used to overcome the problem: chemical derivatization and coordination ion spray-MS. In a recent paper the performance of the two approaches was compared (Bai et al., 2010). In this paper, dimethylamine is used as a derivatizing agent, thus introducing an ionisable or even permanently charged moiety which provides enhanced detectability. On the other hand, coordination ion spray-mass spectrometry (CIS-MS) converts neutral compounds into charged neutral-ion complexes thus increasing detectability. Electron Capture Detector (ECD), due to its selectivity and sensitivity to halogen-containing substances, can be utilized for GTI analysis, especially for alkyl halides (Elder et al., 2008a; Li and Sluggett, 2005). Element specific detectors such as CLND and HECD can also be useful tools in GTI analysis, adopting LC or GC for separation. The Chemiluminescent Nitrogen Specific Detector (CLND) (Corens et al., 2004) can be used both as a stand-alone detector or in conjunction with MS. Its advantages are the sensitivity and its response, that is proportional to the number of nitrogen atoms present in the analyte. The Hall Electrolytic Conductivity Detector (HECD) can be applied for specific and very high sensitivity detection of nitrogen, sulfur and halogen-containing compounds (McCarthy et al., 2005). Due to sensitivity requirements in GTI analysis, only in few cases Evaporative Light Scattering Detector (ELSD) (Douville et al., 2006; Ganzera and Stuppner, 2005) is adequate for a quantitative analysis in the low ppm range (Yuabova et al., 2008). Quantitative NMR (Holzgabre et al., 2008) in some cases can be used as a stand-alone technique, since multiple components can be selectively detected without a chromatographic separation (Holzgrabe et al., 2005; Schulè et al., 2010). This can be helpful to avoid issues related to the isolation of the GTI. An example of NMR-based impurity measurement without separation is the assessment of 4-aminophenol in paracetamol at a level lower than 0.05% (Forshed et al., 2002). The NMR technique can also be used in conjunction with separation systems such us HPLC or capillary electrophoresis and micro-separation techniques (Pan et al., 2006; Alexander et al., 2006; Kuhnle et al., 2009; Yokoyama et al., 2000). In addition to the elimination of interference, hyphenated NMR techniques can increase sensitivity, by combining multi-dimensional liquid chromatography with SPE technology for isolating, enriching and delivering the analyte to the NMR probe. The problems related with the quantitative analysis of mixtures using hyphenated NMR techniques were reviewed in the case of natural product analysis (Pauli et al., 2005). Though promising, the NMR technique has not been extensively applied to the GTI analysis so far. Sensitivity and availability in production QC labs can still be problems with this technique.

6.3. Separation and selectivity

To achieve an appropriate selectivity is the other important task in GTI analysis. Interferences can be due to the main component or to the other genotoxic and non-genotoxic impurities. GTI can be divided on the bases of their volatility and LC or GC separation techniques can be used, respectively, for non-volatile or volatile compounds. LC techniques include HPLC, UPLC and HILIC. Though reverse-phase (RP) chromatography is widely used, low molecular weight and polar GTI are often not appropriately retained by RP-columns. Derivatization could be a solution in this case, for example arylamines and aminopyridines are converted into the corresponding hexylcarbamates to improve the analyte retention (Vanhoenacker et al., 2009). Alternatively, for polar low molecular weight GTI a suitable option is HILIC (Hydrophilic Interaction Liquid Chromatography). Using HILIC for separation and CLND for detection, a method was validated for the assessment of hydrazine, 1,1-dimethylhydrazine and 1,2-dimethylhydrazine in a pharmaceutical product with a LOD in the 200 ppm range (Liu et al., 2009). A selective and sensitive method using HILIC and ESI was described for assessment at low levels of a polar drug (Park et al., 2008). HILIC-UV was also found to be a valuable and sensitive method for the analysis of very low levels of aromatic amines (Li et al., 2010; Gianotti et al., 2008). A very sensitive method for the determination of sulfonate and sulfate esters, which involves

derivatization of these GTI to the highly polar quaternary ammonium salts and separation from the drug substance by means of HILIC, was also reported (An et al., 2008). Furthermore, a method which used a combined HILIC/RP-HPLC system to broaden the elution window was described (Louw et al., 2008). GC or headspace-GC (HS-GC) are generally the preferred methods for the analysis of volatile GTI. The widely used detector for GC is the flame-ionization detector (FID), due to its simplicity of use. However sensitivity is sometimes not appropriate for measuring trace levels of GTI. MS-detectors are the preferable option also in this case. For thermally stable volatile GTI, the HS-GC can be used (Kolb and Ettre, 2006), due to the simple elimination of the matrix. This technique, based on the partitioning of volatile analytes between the headspace gas volume and the liquid matrix sample, is extensively used for GTI analysis. A review focusing on the determination of alkyl and aryl halides by HS-GC in various drug substances was published (Elder et al., 2008a), as well as the use of HS-GC methods to assess sulfonate esters (Elder et al., 2008b). Direct liquid injection is not generally utilized since the drug (matrix) can accumulate in the injector/head of the column, thus affecting the overall performance of the method. A two-dimensional GC method utilizing a Deans switch heart-cutting in combination with MS detection was recently reported as a means to overcome this problem (David et al., 2010). A concentrated solution of the drug substance was directly injected in the GC–MS system using an apolar column for first dimension separation. The fraction (heart-cut) containing the GTI was then transferred into a capillary polar column which allowed for independent temperature-programmed analysis. Direct liquid injection GC methods can be useful when the GTI is derivatized and the volatile analyte is extracted from the matrix by liquid-phase extraction or solid phase micro-extraction (SPME). For example, amines can be derivatized with pentafluorobenzaldheyde and then analyzed by GC–MS (Chiang and Huang, 2008). *In situ* derivatization of hydrazine with acetone and analysis by HS-GC/MS provide a method for the assessment of hydrazine at low ppm levels (Sun et al., 2009). Conversion of the reactive epichlorohydrine into the more stable oxolane, by reaction of the epoxyde with cyclopentanone and GC/MS analysis, provides a method for epichlorohydrine analysis with good sensitivity, precision and recovery (Sung et al., 2008).

6.4. Sample preparation

The appropriate elimination of the matrix during sample preparation can dramatically improve sensitivity and minimize interferences in GTI analysis. Sample preparation has an outstanding importance, since it can concentrate the analyte to adequate levels for measurement, turning out to be as fundamental to the success of the analysis as separation and detection are (Gorog, 2006). The elimination of the matrix can be achieved by liquid–liquid extraction or using its miniaturized version LPME (liquid-phase microextraction) which is faster (Psillakis and Kalogerakis, 2003). During concentration two main problems can occur: the co-distillation of the impurity and its degradation; thus the preparation of the sample should take these issues into account. Matrix elimination by solid phase extraction (SPE) (Baltussen et al., 2002; Pichon, 2000) is an efficient and rapid technique which can be automated (Kuklenyik et al., 2004) and applied for analysis of non-volatile GTI (Kondo et al., 2006). Solid phase micro-extraction (SPME) is also widely used (Kataoka, 2004). In some cases the matrix itself is the cause of instability of the analytes. This instability can be due to the reaction of the analyte with substances present in the matrix. Quenching the reactivity of these interfering substances, able to cause the analyte decomposition, is an approach known as matrix deactivation. Strategies such as protonation and scavenging used to avoid matrix induced decomposition were reviewed (Sun et al., 2010a,b).

6.5. Derivatization

GTI are often reactive substances and this can be an outstanding problem, adding to other features of these analytes, such as volatility, poor chromatographic characteristics, poor solubility, and detectability. Derivatization can provide good opportunities to enhance the analyte stability, to facilitate its separation from the matrix and to enhance sensitivity. Some examples of derivatization have been discussed above. A further example is provided by the assessment of formaldehyde in drug substances (Argentine et al., 2007). Formaldehyde is a very reactive and volatile compound. It can be analysed by a quantitative trapping in the dihydropyridine moiety by reaction with acetylacetone and ammonium chloride. This increases detectability and allows to overcome reactivity and stability problems. The method was used to assess formaldehyde presence in fluoxetine hydrochloride, where it is an impurity of the key intermediate (PMAP; 1-phenyl-3-methylamine-propanol). In this case the assessment is even more complex, since formaldehyde can react with the intermediate giving rise to a derivative (oxazine) which can in turn be a source of formaldehyde. The method was able to detect both the free or the oxazine trapped formaldehyde (LOD about 1 ppm), (Pierson et al., 2009).

7. Quality by design and the control of genotoxic impurities

GTI arising from synthesis, such as reagents, intermediates and by-products can be carried over to the final product. Limits for these impurities in the drug substance represents a challenge for both analytical method development and routine analysis during manufacturing. In addition to the sensitivity problems related to GTI assessment in the low ppm range, these impurities may be reactive or unstable causing additional analytical problems. Though hyphenated-MS analytical methods combined with an appropriate sample preparation can overcome the issue, these methods are often difficult to implement in QC labs at manufacturing sites. In order to overcome these problems it is necessary to devise a strategy to appropriately address the GTI issue during manufacturing. Quality by Design (QbD) may represent the solution (Hussain, 2005; Yu, 2008). The QbD approach is described in the guidelines: ICH Q8 (R2), ICH Q9, ICH Q10 and in the corresponding Question & Answer document. ICH Q8 guideline focuses on the pharmaceutical development of new drug products. It provides the following definition of QbD: "a systematic approach to development that includes incorporation of prior knowledge, results of studies using design of experiments, use of quality risk management and use of knowledge risk management throughout the life cycle of the drug product". ICH Q8 describes an enhanced approach by the use of process understanding whereby process performance is considered over a range of material attributes, manufacturing process options and process parameters. ICH Q9 discusses quality risk management and the tools that can be used to cope with the risk. Finally, ICH Q10 introduces the concept of control strategy, defined as a set of controls derived from product and process understanding, which assures process performance and product quality.

Hence, QbD is a systematic and scientific approach, which utilizes an in-depth understanding of both the product and the manufacturing process, along with the use of appropriate control strategies, to guarantee a consistent quality of the product. According to this approach, a system for designing, analyzing and controlling manufacturing processes is developed. The identification of the variables affecting product quality, the knowledge of risks involved in the manufacturing and how to mitigate these risks are the other fundamental topics QbD is based on. Quality is then assured by an integrated control strategy consisting of suitable pro-

cess and analytical controls which guarantee the product quality (Pharmaceutical Quality for 21st Century: a Risk-Based Approach, http://www.fda.gov/ceder). Process Analytical Technology (PAT) is often indicated as the framework to apply QbD to manufacturing processes. The term PAT is used to describe "a system for designing and controlling manufacturing through timely measurements (i.e. during process) of critical quality and performance attributes for raw and in-process materials and also processes with the goal of ensuring final product quality". "This is in accordance with the fundamental principle that the quality cannot be tested, but is instead built into the medicinal product by design (EMA, Process Analytical Technology)." The concept to assure quality not just by final product testing, but using an integrated control strategy can also be applied to the GTI issue. In this case, in addition to the identification of the GTI which can be formed in the manufacturing, it is also important to understand: (a) which are the process parameters that control formation/removal of these impurities; (b) which are the operating ranges of these parameters that consistently assure the product quality; (c) how to guarantee product quality by an appropriate monitoring of the concentration of these impurities during manufacturing. Using this strategy the presence of a GTI above the appropriate limits in the drug substance can be excluded by controlling critical process variables and with appropriate in-process controls (IPC). This approach can be applied during early development by the identification of appropriate LC–MS methods. These methods will be characterized in terms of specificity, linearity, recovery and sensitivity, in order to guide process development by means of the identification of steps and procedures to properly control each GTI. Optimization of the process purging procedures for the GTI along with the LC–MS assessment can allow for the identification of crucial steps where analytical control of the GTI, by UV–HPLC (or by a limit test) at 1.0–0.1% levels, can exclude its presence in the final drug substance. Thus, analytical methods aimed at assessing the GTI concentration at each step of the process and suitable for a production environment can be developed, along with an appropriate process-tuning aimed at minimizing GTI formation or presence**.** In order to assess the process robustness in terms of its efficiency in the GTI removal, the impurity can be added at an established concentration (spiking) at the appropriate step. The evaluation of the corresponding GTI content in the drug substance highlights the process purging capability. Consequently, a suitable limit can be introduced at a defined step for the GTI. This approach based on process understanding, spiking/purging studies and batch analysis, along with the identification of control points in the synthetic process, enables GTI to be routinely monitored by IPC at % levels instead of at ppm levels in the drug substance, therefore, simplifying drug substance testing and increasing robustness of both the manufacturing and release processes. Examples of implementation of the QbD concepts to the manufacturing of drug substances appeared in several recent papers (Watson et al, 2007). Relevance of this approach to the assessment of GTI was also discussed in a review by Liu (Liu et al., 2010). Testing and control strategies were reviewed in order to identify appropriate methods, with simplified analytical instrumentation and higher detection limits, to be transferred to manufacturing sites. The analytical work done for controlling GTI during the synthesis of a drug (Pazopanib hydrochloride) recently approved and launched in US was reported (Liu et al., 2008). In Pazopanib hydrochloride process five GTIs were identified among the starting materials, reagents and intermediates by a risk assessment on the synthetic route. Among them, one was the known genotoxin dimethylsulfate, the second was an early intermediate identified as GTI by Derek, two of them were found to be positive in the Ames test and finally a late intermediate was found to be a GTI, endowed with a threshold-related mechanism. Limits for these GTI in the drug substance ranged from 1.7 to 115 ppm. After the development of five LC–MS methods for testing these GTI in the drug substance, the concentration of the impurities at each step of the manufacturing process was studied. In addition, a comprehensive spiking/purging study allowed for the identification of control points in the process, suitable for an HPLC/UV detection of the GTI. Thus, by establishing suitable limits (0.1–0.6%) for GTI concentration at the appropriate step, along with an extensive documentation of the manufactured batches, it was possible to demonstrate that the drug substance fully complied with regulatory requirements for GTI. Another example reports the work done for a fluoroarylamine manufacturing process, where sulfonate esters can be potentially formed (Cimarosti et al., 2010). In this process the drug substance is isolated as methanesulfonate salt. The risk assessment on the synthetic route highlighted the possible formation of three GTI (methyl, ethyl and isopropyl methanesulfonate, respectively, MMS, EMS and IMS). Though sulfonic acid is widely used in the preparation of salts of drug substances, the Viracept issue highlighted how sulfonate esters can be easily formed (Elder et al., 2010). These highly reactive esters are GTI and should be controlled in the drug substance at the TTC level. The risk assessment highlighted how these impurities could be introduced in the process at the last step, when the methansulfonate salt of the product is precipitated. Both MMS and EMS can be methanesulfonic acid impurities, in addition these GTI can be formed by reaction of the acid, respectively, with methanol and ethanol. Though methanol and ethanol are not used in the process, they could be introduced as contaminants of the solvents or formed as side-products during reaction/work-up. IMS can be formed by reaction of isopropanol with methanesulfonic acid. Isopropanol is used in the manufacturing of an intermediate and can be an impurity of the solvent used in the last step. The last step involves three operations: crystallization, isolation/washings and drying. The parameters that control the GTI formation/purging during these operations were studied. For the crystallization two groups of parameters were taken into account, those specifically related to the GTI formation and those affecting the co-precipitation of these impurities. The crystallization was then studied according to a multivariate analysis taking into account: (a) the ratio of methanesulfonic acid/base; (b) the concentration of the alcohols; (c) times and temperatures used for the operations (seeding, anti-solvent addition, aging); (d) the ratio solvent/anti-solvent. Moreover, forcing conditions were included in the evaluation in order to assess the robustness of the process. The GTI level was measured both in the suspension and in the precipitated product with the appropriate sensitivity (LOQ < 1 ppm). The experimental results highlighted that the crystallization step did not produce the GTI, when operated within the proposed ranges. Concentrations of the alcohols up to 0.2% (w/w) during crystallization did not give rise to formation of the GTI (levels below 1 ppm). In addition, spiking studies demonstrated that the GTI can be removed during the isolation/washing even when present at a concentration of 1200–2600 ppm. The GTI were not formed during drying, at the established temperature, even when the alcohols were present in excess. This process knowledge provided the rationale to substitute the final product testing for the GTI with an appropriate upstream testing. Thus, specifications for MMS and EMS content in methanesulfonic acid and for the content of the alcohols in the process solvents, along with a limit (IPC) for the concentration of the alcohols in the final crystallization medium, turned out to be the appropriate controls to exclude GTI formation during crystallization. The analysis of the washings of the isolated product and the monitoring of the drying temperature assured the absence of the GTI during isolation/drying. Jointly these controls guaranteed the final product compliance with regulatory limits for the GTI. A further example is focused on the catalytic hydrogenation of a nitroaromatic compound (Looker et al., 2010). In this case the manufacturing of

the drug substance involved the hydrogenation of a nitroaromatic group for the preparation of the key intermediate. Hydrogenation of nitroaromatic compounds can potentially give rise to nitroso and hydroxylamine derivatives as side-products. Both these derivatives highlighted structural alerts. The analytical assessment of these PGI, in several batches of the key intermediate demonstrated their absence (<0.03%) highlighting these as potential impurities. In order to definitely exclude the presence of these impurities in the drug substance, the critical conditions for their formation and for process purging capability were assessed. The study pointed out, among the several parameters investigated, the catalyst/product ratio, the reaction temperature and time during hydrogenation as the parameters affecting the formation of these impurities. A multivariate analysis carried out on these parameters established the reaction time as the most critical one for controlling the formation of these PGI. Once identified the critical process variable to be controlled and the suitable IPC to guarantee the appropriate key intermediate purity, the process purging capability was then evaluated. A LC–UV method able to detect these impurities in the drug substance at the target level (<1 ppm) was developed. Then, by spiking the key intermediate with the PGI, it was possible to demonstrate that process purging capability allowed for removal of these impurities, even when present in the intermediate at levels exceeding the specification level (<0.03%). Thus, controlling one process variable and the intermediate with an IPC, it was possible to exclude the presence of these PGI in the drug substance.

8. Conclusions

The problem of controlling GTI in pharmaceuticals gave rise to additional challenges to the several ones already present in drug development. An inappropriate approach to this issue could dramatically hamper the development of new drugs and, ultimately instead of being beneficial to the patient health, it could have a negative impact on the overall life expectancy and quality of life, due to the reduction of available therapies. On the other hand, actions must be undertaken to prevent GTI presence at a level which could cause health risks which are not commensurate with the benefits expected by the therapy. Despite the regulatory effort that made available the EMA and FDA guidelines, addressing the GTI issue during development of a NCE is still a challenge. Identification and genotoxic potential assessment for these impurities, as well as establishing corresponding limits can constitute a tricky task. The need of controlling several GTI at the TTC level during analytical and process developments further complicates the problem. In big companies the availability of resources and specialists in the several involved disciplines, as well as of suitable instrumentation may adequately face the complexity related to the GTI issue. Conversely, in smaller companies it could be more challenging to manage the GTI topic appropriately. In this review, we strived to provide a summary of the different aspects related to this subject, in order to make available the necessary information to appropriately manage the GTI issue. As discussed above, the problem should be addressed during early development, focusing on the knowledge of both the product and its synthetic process, by means of multidisciplinary competences (Process and Analytical Chemistry, Toxicology and Regulatory). The identification of GTI can be achieved using literature data, structural alerts, and specialized software. When it is deemed as opportune, an appropriate toxicological study to confirm the genotoxicity highlighted by alerting structures or by the Ames test should be performed. During early development, for those cases where several GTI are used or formed in the manufacturing process, it could be wise to take the option for an alternative process into account. Scientific judgement is required to balance the potential for impurity formation and carry-through, with consideration of the risk that can be caused by the presence of the GTI in the drug substance. Limits for the impurities will then be established using available toxicological data or the TTC. On the basis of these limits, suitable analytical procedures to assess the presence of the GTI in the drug substance and during the manufacturing process can be developed. QbD can provide useful solutions both to address analytical and process issues. A study to assess which reaction conditions can control the GTI formation is essential during development. Then, the evaluation of the potential to carry a GTI through to the drug substance becomes crucial, along with the development of appropriate purification procedures for minimizing the GTI concentration in the final product. When the GTI scenario is well established, suitable analytical methods are available, and several batches of the drug substance have been manufactured, a more in-depth study focusing the limits can be addressed. Though for drug substances to be used in clinical phase I and II, the effort necessary to reduce GTI content at the staged-TTC level can be commensurate with the purpose, in advanced clinical development and for commercialization it maybe not be feasible to reach the TTC limit for some GTI. In these cases a more in-depth toxicological study can be necessary in order to assess whether a substance-specific limit can be identified. Finally, we would like to stress that if the QbD approach is applied starting from the early development, it will be possible to consistently control every GTI at the appropriate limit.

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Glossary of the recurrent acronyms used in this review

ALARP: as low as reasonably practicable
EDQM: European Directorate for the Quality of Medicines & HealthCare
GTI: genotoxic impurity/ies
GC: gas chromatography
GC–MS: gas chromatography–mass spectroscopy
HILIC: hydrophilic interaction liquid chromatography
HS-GC: headspace gas chromatography
IPC: in-process control
LC: liquid chromatography
LC–MS: liquid chromatography–mass spectroscopy
LOD: limit of detection
LOEL: lowest-observed-effect-level
LOQ: limit of quantitation
MTD: maximum tolerated dose
NCE: new chemical entity
NOEL: no-observed-effect-level
PAT: process analytical technology
PDE: permitted daily dose
PGI: potential genotoxic impurity/ies
QbD: quality by design
QC: quality control
SAR: structure–activity-relationship
TDI: total daily intake
TGR: transgenic rodent
TTC: threshold of toxicological concern
QSAR: quantitative-structure–activity-relationship
UV–HPLC: high pressure liquid chromatography with ultra-violet detector
WHO: World Health Organization
WOE: weight-of-evidence